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TITLE: The Role of Telomeric Repeat Binding Factor 1 (TRF1) in Telomere Maintenance and as a Potential Prognostic Indicator in Human Breast Cancer

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14. ABSTRACT
The aims of this study are to (i) determine the relationships between the telomere binding protein Telomere Repeat Binding Factor 1 (TRF1) and other telomere binding proteins, (ii) establish the potential of TRF1 as a surrogate marker for telomere content (TC) and as a potential clinical marker and (iii) characterize the relationship between of the telomere binding protein TRF1 and TC. Through examining the role of TRF1 in telomere length control and in breast cancer progression, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, all tasks; as outlined in the Statement of Work, are completed.

15. SUBJECT TERMS
HUMANBREASTCANCERTISSUE,TELOMERES,TELOMERE-BINDINGPROTEINS, IMMUNOHISTOCHEMISTRY, RT-PCR, SLOT BLOT, SIRNA

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I. INTRODUCTION

The aims of this study are to determine the relationship of the telomere binding protein TRF1 to other telomere binding proteins, establish the potential of Telomere Repeat Binding Factor 1 (TRF1) as a surrogate marker for telomere content and as a potential clinical marker and further, to characterize the relationship of the telomere binding protein TRF1 to telomere content. Through examining the role of TRF1 in telomere length and in breast cancer, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, Specific Aim 2 has been completed and a manuscript based on the data is being edited for submission. The student has received a no cost extension, which should allow the completion of Aim 1 and 3. Three of the tasks in Specific Aim 1 were delayed due to a technical issue, however this issue has been overcome and the data collection is in progress and expected to be completed prior to the end of the extension. Several of the tasks Specific Aim 3 were delayed due to necessary changes in techniques, however the new methods have been trouble shot and data collection is in progress. All Aims are expected to be completed by the end of the no cost extension.

Hypothesis and Rationale

Telomere Content (TC) has prognostic value in breast cancer; however the factors that control TC are poorly understood. In vitro studies have shown that high levels of TRF1 can stabilize short telomeres and the preliminary results suggest the levels of TRF1 mRNA are related to TC. Together this data suggests that TRF1 level may be directly related to telomere content and therefore be a potential biomarker for telomere length. However, TRF1 also has multiple interacting partners, TRF1 Interacting Nuclear Factor 2 (TIN2), Tankyrase, Telomere Repeat Binding Factor 2 (TRF2) and Protection of Telomeres 1 (POT1), which may modify the interaction between TRF1 and TC. If increases in TRF1 are partially responsible for decreased TC, a prognostic marker of poor outcome, then targeting TRF1 may be a good preventive treatment of breast cancer progression. However, it is also possible that the observed increase in TRF1 is a cellular reaction in response to low TC and therefore a good surrogate for TC. Theses two scenarios must be tested to evaluate the prognostic significance of TRF1 in human breast cancer. Therefore *I hypothesize that defining TRF1 levels using immunohistochemistry could provide a surrogate measure for TC that would be easily adaptable to the clinical setting*. To test this hypothesis I will assess the potential prognostic value of the TRF1 in human breast tumor samples. Additionally, I propose to characterize the relationship of TRF1 to TC, and to TIN2 and Tankyrase to further examine the relationship of TRF1 to TC. I will evaluate this hypothesis through three specific aims.

- **Specific Aim #1**

  ASSESS THE POTENTIAL OF TRF1 PROTEIN LEVELS AS A SURROGATE FOR TELOMERE DNA CONTENT (TC) IN FROZEN AND PARAFFIN EMBEDDED BREAST TUMOR TISSUES.

- **Specific Aim #2**
  Assess the potential modification of the relationship between TC and TRF1 mRNA levels by TRF1 interacting protein 2 (TIN2) and Tankyrase in frozen human breast tumor samples.

- **Specific Aim #3**
  Examine the effects of increased TRF1 expression on TC and decreased TC on TRF1 expression in breast cancer cell lines.

II. KEY RESEARCH ACCOMPLISHMENTS

IIa. RESEARCH ACCOMPLISHMENTS

- There exists an association between the levels of TRF1, TIN2 and POT1 mRNA within breast tumors, as measured by real-time RT-PCR.
- The levels of TRF1mRNA are not associated with the mRNA levels of the human telomerase reverse transcriptase (hTERT) mRNA or the levels of TRF2 mRNA within breast tumors.
- The levels of TIN2, TRF1, TRF2 and POT1 mRNA are all associated with telomere content.
- Visualized TRF1 and TRF2 distribution by Immunohistochemistry.
- Developed siRNA for hTERT.
- Developed TRF1 overexpression vector.
- Developed 2 drug treatments to increase TRF1, TIN2 and POT1 mRNA levels
- Developed a drug treatment which decreases telomere content without changing hTERT levels
- Assessed the relationship of TRF1 and TRF2 to cell cycle by flow cytometry
- Developed a western blotting procedure for TRF1 and TRF2 proteins
- Demonstrated a correlation between TRF1 and TRF2 protein level and mRNA level
- Demonstrated an inverse relationship between TRF1 and TRF2 protein levels and TC
- Demonstrated a decrease in TC can lead to an increase in telomere protein mRNA levels in breast cancer cell line models
- Demonstrated and increase in TRF1 and it's associated proteins can lead to a decrease in TC

IIb. TRAINING/EDUCATIONAL ACCOMPLISHMENTS

Since the previous annual review, the PhD candidate has had continuing opportunities to work and interact with oncologists, pathologists and other PhD scientists who specialize in breast cancer. These interactions have occurred though tumor board meetings, journal clubs, special seminars and direct interaction within the laboratory. To the training in microscopy, cryosectioning and paraffin sectioning she received in the first year and second years of the award, she has continued to receive training in flow cytometry from the UNM Flow Cytometry. On an educational level, the candidate is in final preparations for completion of her doctoral degree, to be completed summer of 2008. The candidate has aspirations of continuing her career in research and remaining in academia and felt teaching provided an opportunity to develop the essential teaching skills need for her chosen career.

IIc. PERFORMANCE ACCOMPLISHMENTS:

Experimental Milestones

Specific Aim 1: (7 tasks) Completed

Task 1 *Month 1-2* Completed in year 1

- Purify DNA from paraffin embedded breast tumor samples previously collected by our laboratory.

Task 2 *Month 2-6* Completed in year 1

- Measure TC in paraffin embedded breast tumors samples.

Task 3 *Month 6-12* Completed in year 1

- Optimize TRF1 antibody for use in frozen tissue and paraffin embedded breast tumor tissue.

  TRF1 antibody specificity has been demonstrated in breast cancer cell-line MCF-7 and conditions for antigen retrieval and staining have been determined.

Task 4 Month 12-14 **Completed**

- Section frozen human breast tumor samples and stain with antibody to TRF1.

  Following the transition from immunohistochemical staining of TRF1 to western blot, the student has completed western blot analysis of 37 frozen breast tumor samples.

Task 5 Month 13-14 Completed in year 2

- Assess relationship between normalized TRF1 mRNA levels and TRF1 staining intensity.

  Student demonstrated that the levels of TRF1 mRNA as measured by quantitative real-time PCR relate to the staining intensity of TRF1 visualized in various breast cancer cell lines by immunohistochemical staining.

Task 6 Months 14-24 Modified

- Section paraffin embedded breast tumor tissues and stain with antibody to TRF1.

  TRF1 and TRF2 staining has been shown to be dependant on the cell cycle, precluding its use as a clinical immunohistochemical stain. The student has decided to answer the question of the relationship of TRF1 and TRF2 to clinical markers and

TC by western blot. Western blot is highly unreliable in paraffin embedded tissue so the student will focus on frozen tissue.

Task 7 Months 12-30 **Completed**

- Score sections stained with TRF1 antibody and compare to TC data, histological markers and survival data.

Due to the relationship of TRF1 to cell cycle, which precludes the use of TRF1 as a clinical immunohistochemical stain, the student has decided to determine the protein level of TRF1 and TRF2 by western blot. Student's work has demonstrated a direct relationship between protein level of TRF1 and TRF2 as assessed by western blot and mRNA level as previously assessed by RT-PCR. A direct and inverse relationship has also be demonstrated between TC and the TRF1 and TRF2 protein levels

Specific Aim 2: (4 tasks) Completed

Task 1 *Month 1-2* Completed in year 1

- Extract RNA from frozen breast tumor samples already collected by our laboratory. Design and order Tankyrase and TIN2 primers and probe.

RNA was extracted from 36 breast tumors. Primers for Tankyrase and TIN2 were designed.

Task 2 *Month 2-4* Completed in year 1

- Optimize Tankyrase and TIN2 RT-PCR

TIN2 RT-PCR was optimized, however Tankyrase primers picked up both Tankyrase 1 and the analog Tankyrase 2. The expression levels of these two proteins are quite different and Tankyrase 2 is highly expressed and functionally not associated with telomere management. Assessment of Tankyrase by RT-PCR yielded an inconclusive result in all experiments. RT-PCR experiments to determine Tankyrase mRNA levels have been placed on hold pending new methods to delineate these two analogs at the molecular level. Recent studies have determined the regulation of Telomere length by proteins to involve a number of complexes, which include TRF1 and TIN2, and also include Tankyrase, POT1 and TRF2. As levels of Tankyrase mRNA could not be determined and POT1 and TRF2 levels may be associated with TRF1 mRNA levels and involved in telomere content determination, RT-PCR reactions were optimized for TRF2 and POT1 as well.

Task 3 *Month 4-7* Completed in year 1

- Measure Tankyrase and TIN2 mRNA levels by RT-PCR in RNA extracted from frozen breast cancer samples.

Tankyrase mRNA levels could not be assessed; however TIN2, POT1 and TRF2 mRNA levels were assessed in 36 frozen breast tumor samples.

Task 4 *Month 7-12* Completed in year 1

- Analyze association between Tankyrase and TIN2 mRNA levels with TC and TRF1 mRNA expression.

  Tankyrase mRNA levels could not be assessed so no comparison was possible. TIN2 mRNA levels showed a strong association with TC and TRF1 levels as well as two other telomere binding proteins; POT1 and TRF2.

Specific Aim 3: (6 tasks) Near Completion

Task 1 *Month 12-15* Completed in year 2

- Design and test small interfering RNAs (siRNA) of human Telomerase Reverse Transcriptase (hTERT)

  The candidate designed several siRNAs against hTERT and tested these siRNAs. The siRNAs showed a reduction in hTERT expression by quantitative real time PCR.

Task 2 Month 15-27 **Completed**

- Express siRNA of hTERT in breast cancer cell lines and examine TRF1 mRNA levels and TC by RT-PCR and slot blot over time.

  Student used the drug, N,N′-*bis*[2-(1-Piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic Diimide (referred to as PIPER) to cause rapid telomere attrition without changing hTERT levels. The student then completed long-term exposure of 3 breast cancer cell lines to PIPER. After treatment, the student demonstrated significant decreases in TC, no change in TERT levels and increases in the mRNA levels of TRF1 (Appendix A).

Task 3 Month 15-18 Completed year 2

- Design, generate and test TRF1 expression vector.

  Student has designed and generated a TRF1 expression vector, which demonstrates an increase in TRF1 mRNA levels when examined by quantitative real time PCR.

Task 4 Month 18-30 **Completed**

- Overexpress TRF1 in breast cancer cell lines and examine TC levels by slot blot over time.

Previous experimentation completed in Specific Aim 2 has demonstrated that the level of TRF1 is coordinately regulated with the telomere-associated proteins TIN2 and POT1. Therefore, to examine the effects of changes of TRF1 on TC levels, it is necessary to change all three protein levels at the same time. To do this, the student examined the use of drug treatments to increase these three mRNA levels and has identified two drugs, Dexamethasone and TNF-alpha, which selectively increases TRF1, POT1 and TIN2 without increasing TRF2 or hTERT. The student treated 3 breast cancer cell lines with these drugs and collected populations to examine the relationship of TRF1, POT1 and TIN2 to TC levels. The student demonstrated a selective increase in TRF1, POT1 and TIN2 levels with both drug treatments in all 3 cell lines. Increases in the TRF1, POT1 and TIN2 levels lead to a decrease in TC in all 3 cell lines (Appendix B).

Task 5 Month 30-34 **Completed**

- Analyze relationship between TRF1 and TC.

Long term data collection and analysis are complete. Decreased TC can lead to an increase in TRF1 mRNA levels (Appendix C).

Task 6 Months 30-36 **In Progress**

- Prepare and submit manuscripts.

Candidate is currently in the final editing stage of three manuscripts covering all data collected to date.

Education and Training Milestones (6 tasks)

Task 1 *Month 1-6* Completed in Year 1

- Learn to recognize morphology and features of different types of breast cancer under the guidance of Dr. Nancy Joste.

Student has examined various types of breast cancer and can recognize features of different tumors and tumor stages.

Task 2 Month 1-36 **Completed**

- Attend tumor board meetings and monthly Cancer Research and Treatment Center Meetings to gain understanding of current treatments for breast cancer and ongoing clinical trials.

Student continues to attend tumor board and the Cancer Research and Treatment Center Meetings on a regular basis and has developed a working relationship with several of the doctors to allow further understanding of current treatments and clinical trials in breast cancer.

Task 3 Month 1-6 Completed in Year 2

- Attend the University of New Mexico School of Medicine medical student training Neoplasia block.

  Student attended all the lectures the Genetics and Neoplasia block and also acted as a tutor for the problem based learning sections of the Genetics and Neoplasia block.

Task 4 Month 6-12 Completed in Year 2

- Learn staining procedures and significance of histological markers commonly used in breast cancer under the guidance of Dr. Nancy Joste.

  Student has learned basic staining procedures and has gained understanding of the commonly used markers to determine treatment in breast cancer.

Task 5 Month 12-24 Completed in Year 2

- Work with oncologists in the University of New Mexico Hospital to gain perspective on breast cancer.

  Student has developed a working relationship with several doctors in the Cancer Center and has used these relationships to develop an understanding of patient care and current issues in breast cancer treatment.

Task 6 Months 12-36 **Completed**

- Present ongoing work at local and national meetings

  Student has presented her work in national and local meetings

III. REPORTABLE OUTCOMES

Papers: In preparation

KS Butler, WC Hines, C Fordyce and JK Griffith. "Levels of Telomere-Associated Protein mRNAs Suggest Coordinate Regulation of Their Transcription" 2008

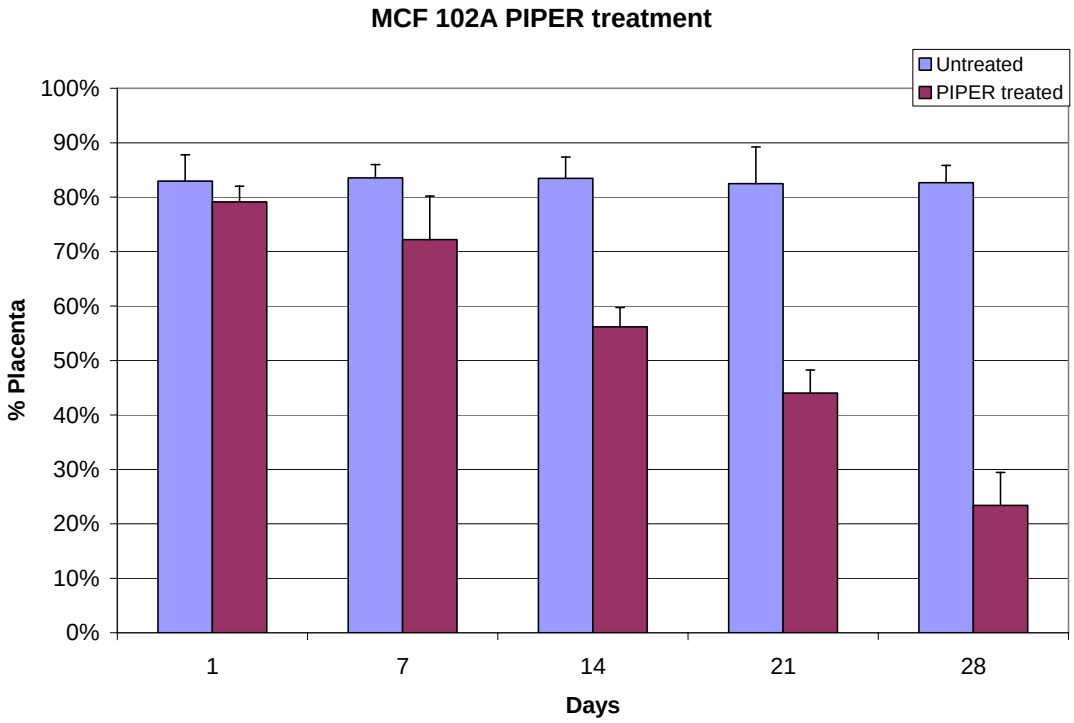
KS Butler, CM Heaphy and JK Griffith "Soluable factors, such as TNFalpha and Dexamethasone, can lead to decreased telomere length" 2008

KS Butler, M Bisoffi and JK Griffith "Decreases in telomere content can lead to increases in telomere protein mRNA levels in cancer cells" 2008

IV. CONCLUSIONS

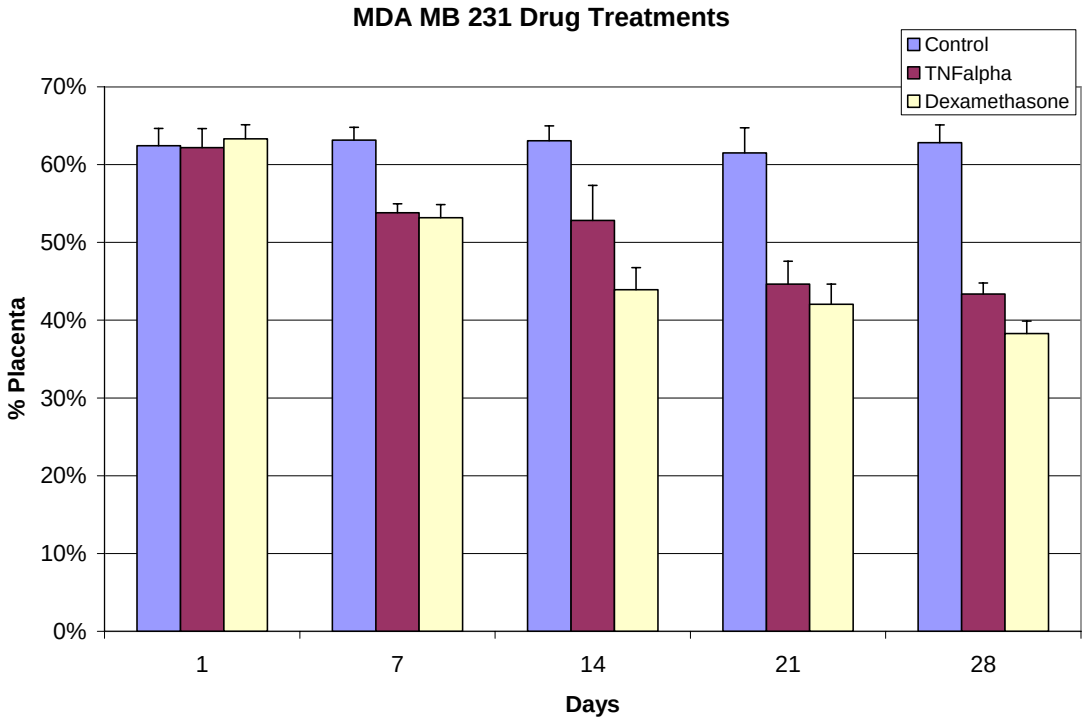
To date, all experimental tasks; as outlined in the Statement of Work are completed. The student is currently preparing three manuscripts for publication based on the research presented above. The PhD candidate has completed her educational goals and will finish her doctoral thesis Summer 2008.

Appendix A



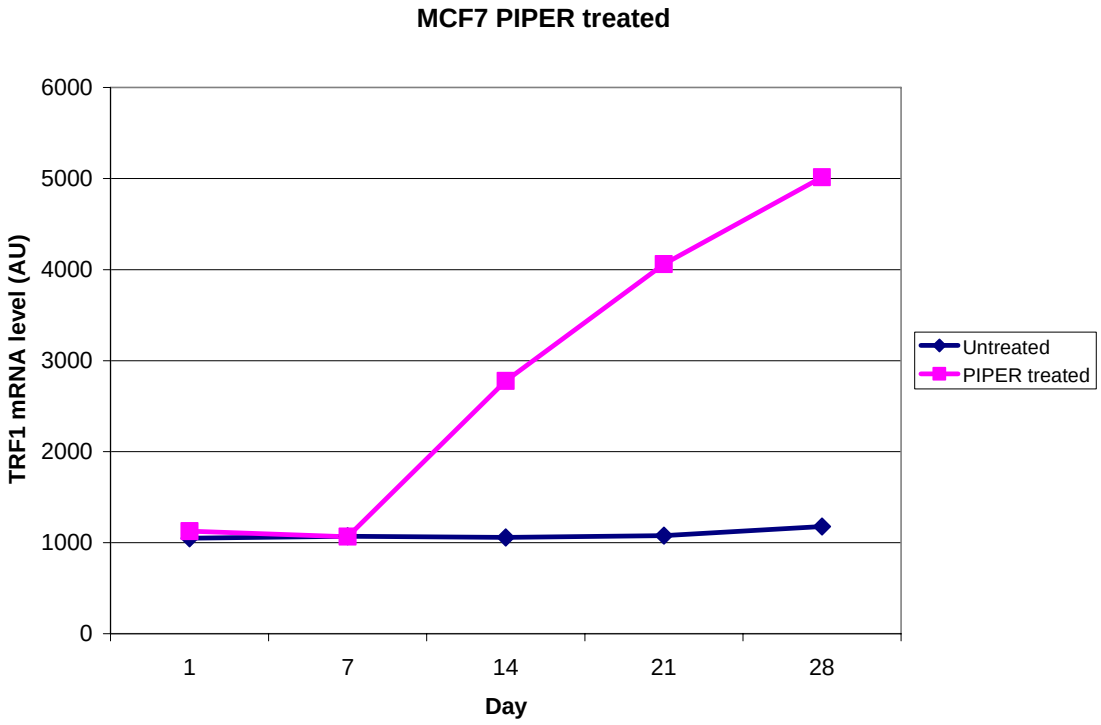
Representative figure of the treatment of breast cancer cell lines with PIPER. MCF 102A cells were treated for 4 weeks with Piper. The blue column represents untreated MCF 102A cells over the treatment time line. The untreated cells showed no change in TC over 4 week's time (shown as a percent of placenta control). The purple columns represent day 1 to day 28, of treatment with PIPER. The PIPER treated samples show a steady decrease in telomere content over the 24 days of treatment.

Appendix B



Representative figure of the treatment of breast cancer cell lines with TNF-alpha and Dexamethasone. MDA MB 231 cells were treated for 4 weeks with TNF-alpha and Dexamethasone. The blue column represents untreated MDA MB 231 cells over the treatment time line. The untreated cells showed no change in TC over 4 week's time (shown as a percent of placenta control). The purple and yellow columns represent treatment with TNF-alpha and Dexamethasone, respectively, over 4 weeks. The Dexamethasone and TNF-alpha treated cells showed a significant increase in TRF1, TIN2 and POT1 mRNA after 24 hours and a significant decrease in TC after 14 days.

Appendix C



Changes in TRF1 mRNA level in response to decreased TC caused by PIPER. MCF7 cells were treated for 4 weeks with PIPER. No changes in TRF1 mRNA levels were noted in the untreated control (blue). The TRF1 mRNA levels of the PIPER treated cells increased between 7 and 14 days which correlates to a significant decrease in telomere content caused by PIPER treatment.